47. (Previously presented) A kit of claim 46, further comprising a mutated EtaA gene for use as a positive control.

48. (Currently amended) A kit of claim 47, wherein said mutated EtaA gene is selected from the group consisting of (a) a mutated EtaA gene comprising a frameshift mutation selected from the group consisting of a deletion at position 65, an addition at position 567, and an addition at position 811, and (b) a mutated EtaA gene comprising a single nucleotide polymorphism which causes an amino acid substitution selected from the group consisting of: G43C, P51L, D58A, Y84D, T186K, T342K, and A381P.

REMARKS/ARGUMENTS

I. Status of the Claims

Claims 1-5, 8-12, 21, 22, 25, 28, 29 and 34-48 are pending. Claims 6, 7, 16, 23, 24, 26, and 27 have been previously cancelled, claims 13-15, 17-20, and 30-33 have been withdrawn as drawn to non-elected inventions, and claims 34-48 were previously added.

II. The Present Amendments

The present amendments add no new matter.

The amendments to claim 1 recite that the claimed method is a method of determining the ability of a *Mycobacterium tuberculosis* (Mtb) bacterium to oxidize ethionamide, thiacetazone, or thiocarlide by detecting an amino acid in an EtaA gene which differs from that of SEQ ID NO:2 by comprising any of 10 different mutations. The recitation regarding the ability of Mtb to oxidize ethionamide, thiacetazone, or thiocarlide is supported throughout the specification, including claim 16 as originally filed. The specific mutations in SEQ ID NO:2 are likewise supported throughout the specification, including claims 2 and 4 as originally filed.

The recitation of claim 21 has been amended to recite that the Mtb are resistant to treatment with ethionamide, thiacetazone, or thiocarlide. The recitation is supported throughout the specification, including page 8, paragraph 23. The claim also now recites that the mutation in the EtaA gene is selected from a group of mutations recited in the claim. The mutations recited are supported throughout the specification, including Figure 4, panels B and C.

Claim 25 has been amended in the same manner as claim 1. The amendments to claim 25 are supported by the passages noted in connection with claim 1.

Claim 34 has been amended to recite methods in which the amino acid sequence of the EtaA gene in an Mtb organism is selected from a group of mutations recited in the claim. The mutations recited are supported throughout the specification, including Figure 4, panels B and C.

The dependency of claim 37 has been changed to reflect the cancellation of claim 36.

Claim 44 has been amended to recite methods in which the amino acid sequence of the EtaA gene in an Mtb organism is selected from a group of mutations recited in the claim. The mutations recited are supported throughout the specification, including Figure 4, panels B and C.

Applicants respectfully submit that entry of the amendments is appropriate at this time as they place the claims in condition for allowance or, alternatively, place them in better condition for appeal.

III. The Office Action

The Action rejects the pending claims on several grounds. Applicants amend in part and traverse. For the Examiner's convenience, the rejections are discussed below in the order in which they are presented in the Action.

A. Rejection of the claims under §112, first paragraph

Claims 1-5, 8-12, 16, 21, 22, 25, 28, and 29 are rejected under 35 U.S.C. § 112, first paragraph, as not enabled. The Action concedes that the specification is enabled for

methods of determining the ability of a *Mycobacterium tuberculosis* ("Mtb") bacterium to oxidize a thiocarbonyl or thioamide found in the drugs ethionamide ("ETA"), thiacetazone ("TA") or thiocarlide ("TC") by detecting one of the mutations exemplified in the specification in the Mtb EtaA gene. The Action contends, however, that the specification does not provide enablement for methods of determining the ability of an Mtb to oxidize any thioamide or any thiocarbonyl by detecting any mutation in the EtaA gene of an Mtb.

In this regard, the Action contains a litany of what it asserts are omissions in the specification. For the sake of good order, the Applicants will respond briefly to each

(1) The specification "omits a teaching of a mutation in every nucleic acid of the EtaA gene that is associated with resistance to all of the drugs in both of the claimed drug classes." Action, at page 5.

Applicants assume that what the Action refers to as "every nucleic acid of the EtaA gene" refers to mutations in the sequence of the wild-type gene, SEQ ID NO:1. Applicants respectfully note that the claims as presented following the last amendment recite mutations in the gene which encode an amino acid sequence which differs from the wild-type amino acid sequence, as set forth in SEQ ID NO:2. It is part of the teaching in the art that Mtb has a low rate of synonymous mutation and that the Sreevatsan et al. study showed that 95% of mutations in Mtb genes were found to be associated with amino acid replacements. Thus, the art of record teaches that there is a high expectation that any mutation in the EtaA gene would result in an amino acid change.

Applicants also respectfully note that one of the reasons the specification warranted publication in PNAS (as previously noted, the specification was the basis for DeBarber et al., PNAS 97(17):9677-6682 (2000)) was that all but one Mtb isolate from patients resistant to ETA with mutations in EtaA were also resistant to thiocarlide, even though the patients had never been treated with this drug. There is therefore also a very high expectation that any particular amino acid change would be associated with resistance to all the drugs in the claimed classes.

(2) The specification "does not teach the way in which each drug . . . differs; nor does it teach the characteristic shared between all drugs responsible for conferring the antibiotic resistance." Action, at page 5.

Applicants observe that the specification indicates that it appears that ETA acts through EtaA-catalyzed S-oxidation (specification, at page 26, lines 14-16 and page 27, lines 20-24), and indicates that "EtaA is directly responsible for thioamide S-oxide oxidative activation." Specification, page 27, lines 20-24. Thus, the specification does provide a characteristic shared by the claimed classes of drugs. Applicants respectfully note that ethionamide, thiacetazone and thiocarlide are all thiocarbonyl drugs, that is, drugs that contain a sulfur double-bonded to a carbon. Applicants note that ethionamide and thiacetazone are also thioamides, since the carbon that is double bonded to the sulfur is also bonded to an NH_2 group. (For the Examiner's convenience, Applicants note that the structure of ETA is shown in the DeBarber PNAS paper of record, on page 9681, as compound 1 of Figure 5, while thiacetazone is shown on page 9680 as compound 11 of Figure 4A.)

Applicants respectfully note that the persons of skill in the art are typically M.D.s and Ph.D.s and can be expected to understand the relationship between thiocarbonyl and thioamide drugs given the specification's teachings about the thioamide S-oxide oxidation activation pathway. Finally, Applicants respectfully note that none of the drugs are "responsible for conferring the antibiotic resistance," and surmise that the Action meant that the specification does not explain why changes in the EtaA gene confer drug resistance. Applicants respectfully maintain that, given the specification's teachings regarding the thioamide S-oxide oxidation activation pathway, persons of skill would assume that changes in the amino acid sequence of the EtaA gene would confer resistance to the drugs by reducing the ability of the organism to catalyze S-oxidation.

(3) "The specification teaches that the drugs, ETA and TA and TC confer different resistance status to the bacterium (Fig 4C). The specification does not teach how or why such supposedly similar thioamide or thiocarbonyl-containing antituberculosis medications confer varying degrees, if at all, of drug resistance." Action, at page 5. The Action cites *In re*

Vaeck as requiring that the specification must teach how to make and use the invention as broadly as claimed and *In re Fisher* as requiring more guidance as the art is less predictable.

Applicants respectfully note that the question is whether a particular Mtb isolate from a patient is susceptible to treatment with a thioamide or thiocarlide. None of the drugs are "responsible for conferring [] antibiotic resistance." Applicants surmise that the Action intended to assert was that the specification does not explain why changes in the EtaA gene confer drug resistance. Applicants respectfully maintain that, given the specification's teachings regarding the thioamide S-oxide oxidation activation pathway, persons of skill would understand that changes in the amino acid sequence of the EtaA gene would confer resistance to the drugs by reducing the ability of the organism to catalyze S-oxidation, as explained in the preceding section. Thus, Applicants maintain that the specification does teach how to make and use the invention as broadly as claimed, and that the invention is not unpredictable. Thus, Applicants maintain that the invention as claimed meets the requirements articulated in *Vaeck* and *Fisher*.

(4) "[O]ne cannot readily anticipate which of the mutations within the gene (i.e. mutations other than those set forth in Table 4C) actually result in the inability to oxidize thiocarbonyl groups and that would be associated with a patient that is resistant to such thiocarbonyl-containing antituberculosis medications, as opposed to those frameshifts or polymorphisms that result in drug sensitivity." Action, at page 6. The Action acknowledges the Sreevatsan el al. teachings regarding the low rate of synonymous mutations in Mtb, but alleges that "the reference does not teach a correlation between the occurrence of amino acid change and specific drug resistances." *Id.* The Action further acknowledges that Sreevatsan teaches "a strong suspicion that the variation has functional significance, such as antibiotic resistance," (Action, at page 6, quoting Sreevatsan), but states that "the reference provides no data relating the amino acid changes to any antibiotic resistance." *Id.* Finally, the Action states that "applicants have not shown that 'every mutation in the EtaA gene will reduce the ability of a Mtb organism to oxidize a thioamide or thiocarbonyl drug, and therefore increase resistance of the organism." Action, at pages 6-7, bridging sentence.

As the Action itself acknowledges, the Sreevatsan reference shows that the art taught, prior to the filing date, that there was a "strong suspicion" that a mutation causing a change in amino acid sequence in Mtb would have "functional significance, such as antibiotic resistance." The Action indicates that this is not sufficient because, it states, the amino acid changes have not been correlated to any antibiotic resistance. Thus, it appears that, to find the claims enabled, the Action would require that the reference have cataloged every mutation of the EtaA gene and its effect on ETA, TA, and TC resistance. Failing that, Applicants presume that the Action would require that the Applicants have mutated each amino acid residue encoded by EtaA and tested the resulting construct for S-oxidation activity. Applicants maintain, however, that the patent laws do not require practitioners to conduct what would be an impossible number of tests. Rather, the standard is whether there is predictability. As noted in the Applicants' last amendment, the Sreevatsan reference sets forth the results of a study of sequence data for 26 genes in hundreds of Mycobacterium isolates. It reports that a "[c]ompilation of the two megabases of sequence data for the 26 genes revealed that greater than 95% of nucleotide substitutions caused amino acid replacements or other mutations in gene regions linked to antibiotic resistance," (page 9870, bottom right), and that "greater than 95% of nucleotide changes were directly associated with antibiotic resistance." (*Id.*, page 9873, left column, second full paragraph, emphasis added.) The authors concluded that "[t]he lack of allelic diversity means that when amino acid polymorphisms, or regulatory region nucleotide variation are observed, there should be strong suspicion that the variation has functional consequences, such as antibiotic resistance." *Id.*, at page 9872, bottom right hand column.

Sreevatsan thus indicates that a mutation in an Mtb gene predictably has "functional significance," and that the functional significance of a mutation of a gene associated with susceptibility to an antibiotic is resistance to that antibiotic. The Sreevatsan reference therefore gives substantial predictability that a mutation that causes an amino acid change in the product of the EtaA gene will cause increased resistance to drugs metabolized by the EtaA gene product.

The correlation between changes in amino acid sequence and changes in resistance is demonstrated in the specification, which shows the uniform effect of changes in amino acid sequence from the wild type sequence in rendering organisms with the mutations resistant to ETA and TA, and 9 out of 10 of the organisms resistant to TC. Thus, the specification shows there is a very high degree of predictability that changes in the amino acid sequence will result in increased resistantance to thioamides and thiocarbonyl drugs.

This predictability is further demonstrated by the post-filing date art. Attached hereto in this regard is a copy of Morlock et al., Antimicrob. Agents Chemother. 47(12):3799-3805 (Dec. 2003). This very recent study characterized the DeBarber et al. PNAS article as showing that "ETH [ethionamide] must undergo activation via an EthA-mediated process . . . Genetic alterations leading to reduced EthA activity would be expected to result in increased ETH resistance." Morlock, supra, at page 3800, left column. Further, Morlock et al. sequenced 41 Mtb isolates collected from the U.S., Russia, and Brazil. Of these, 15 had mutations in the EtaA gene (Morlock et al. uses the terminology of Baulard et al., J. Biol. Chem. 275(36):28326-31 (Sept. 2000), which called the gene "EthA" and ethionamide "ETH"). Morlock, at page 3801, bottom left column. The mutations were all different, and were previously unreported. See, abstract. That is, they are not the same mutations as reported in DeBarber. All of the 15 isolates with mutations in the EtaA gene had minimum inhibitory concentrations (MICs) of 50 μg/ml or higher. Morlock, at page 3802, bottom left column and Table 2. By contrast, no isolate sensitive to ETA had a mutation in the EtaA gene. See, Table 2.

In short, the Applicants showed 9 mutations in EtaA and found increased resistance to ETA, TA, and TC (and, in one case, an isolate which remained susceptible to TC). No mutations were associated with increased sensitivity to these drugs. Morlock et al. found an additional 15 mutations in the EtaA gene, all of which were associated with increased resistance to ETA, the only of the claimed drugs they tested. In full agreement with the expectation set forth in the present specification, no isolates were found which had a mutation in the EtaA gene and which were susceptible to ETA.

Thus, the expectations stated in the specification have continued to be confirmed and expanded by the art since the filing of the present application. Applicants respectfully maintain that the Action fails to set forth a *prima facie* case that there is any unpredictability that mutations in the EtaA gene will not result in increased resistance to ETA or to the other drugs claimed.

(5) "Response to Arguments"

At pages 7 to 10 of the Action, the Action replies to responses made by the Applicants in the previous amendment. For the most part, the Action's replies here echo the assertions already responded to above. For the sake of a complete response, however, Applicants will briefly comment on them.

(a) Comments on Figure 4C of the specification. At page 8 of the Action, the Action explains that the intent of the previous office action was to refer to Strain AS7TAR of Figure 4C of the specification, which shows a Mtb isolate with a mutation in the EtaA gene that shows susceptibility to treatment with TC. The Action maintains that the existence of this example constitutes a teaching that a mutation in EtaA does not confer resistance to all thioamines and thiocarbonyls. Applicants understand that the Action maintains a perfect correlation to support the claim, while Applicants maintain that the results shown demonstrate a high degree of correlation between such mutations and the existence of resistance to the claimed drugs, a correlation which is further supported by the results presented in the post-filing date art cited above. Notwithstanding the foregoing, however, to expedite prosecution, the claims have been amended to recite that the methods pertain to predicting resistance to ethionamide, thiacetazone, and thiocarlide.

(b) Comments on various statements from the previous office action

At pages 8-10, the Action replies to a series of contentions regarding interpretation of the information presented in the specification. The contentions and Applicants' responses, are as follows:

(i) that Sreevatsan does not correlate amino acid changes and any antibiotic resistance, Action at page 9. This contention has been addressed in Section III A (1), above.

(ii) that Applicants have not shown that every mutation in the EtaA gene will reduce the ability of a Mtb organism to oxidize a thioamide or thiocarbonyl, Action, at page 9. This contention has been addressed in Section III A (4), above.

(iii) that the specification does not teach a common property of the drugs that is responsible for the shared resistant phenotype. Action at page 9. This contention is addressed in Section III A (2), above.

(iv) the "specification should clearly assert the exact mechanism by which each of the claimed drugs metabolize the mutant EtaA gene product and in doing so, asserting the common thread shared by all drug classes. It is presently highly unpredictable to assume that all drugs in both of these claimed classes share the same mechanism." Action, at pages 9-10, bridging sentences. Applicants respectfully note that there is no requirement in the patent statute that an applicant have any understanding of the mechanism by which an invention works. There is certainly no requirement that the applicant "clearly assert the exact mechanism" by which the invention works. The Examiner is respectfully reminded that aspirin was known and used for close to a century before its mechanism of action in repressing prostaglandin synthesis was elucidated. Aspirin was and is, nonetheless, a useful product.

Presumably, the Action's contention is meant to convey that in the context of the present invention, it is unpredictable unless the applicant can explain the mechanism shared by the three drugs noted. Applicants note, however, that the isolates analyzed in Figure 4C of the specification were selected because they were from patients resistant to ETA. All but one of the Mtb organisms from these patients were found to be resistant to TC, even though they had never been challenged with TC. See, page 30, lines 13-16. Thus, Applicants respectfully maintain that the specification shows a high degree of predictability that mutations in EtaA are predictive of increased resistance to thioamide and thiocarlide drugs. Notwithstanding the foregoing, to expedite prosecution, the claims have been amended to recite ETA, TA, and TC.

6. Application of the rejections to new claims 34-48

Claims 34-48 were added in the Applicants' last amendment, but recite the specific drugs ETA, TA, and TC, rather than thioamide or thiocarlide drugs generally. Pages 10-15 of the Action apply to these claims the same rejections discussed in detail above. Pages 10-15 assert that, while these claims recite specific drugs, the claims are not enabled for any mutation in the EtaA gene. The Action repeats the arguments set forth and responded to above, including the alleged requirement that the specification teach the exact way each drug differs, the exact class in which the three drugs belong, and what structural motif they have that is responsible for this classification. These arguments have been discussed and responded to above.

The Action further states that the specification fails to teach the "common property represented within each mutation that is responsible for the resulting drug resistance," Action, at page 12, and "how or why such supposedly similar thioamide or thiocarbonyl-containing antituberculosis medications confer varying degrees, if at all, of drug resistance." Action, at page 13. Applicants respectfully note that the medications do not confer degrees of drug resistance. As Applicants noted in response to the previous office action, which contained similarly inaccurate wording, they surmise that the Action meant to convey that the specification does not explain how the mutations in the EtaA gene confer differing degrees of drug resistance to the organisms containing the mutated gene.

Applicants respectfully remind the Examiner that there is no requirement in the patent law that applicants for a patent understand or explain the basis for their invention. Presumably, the present Action recites this contention to explain why it considers it unpredictable that the results of the specification could be extended to other mutations in the EtaA gene. But, as the Applicants noted in their last response, the Sreevatsan reference, already of record in this proceeding, sets forth the results of a study of hundreds of Mycobacterium isolates. The Sreevatsan authors reported that a "[c]ompilation of the two megabases of sequence data for the 26 genes revealed that greater than 95% of nucleotide substitutions caused amino acid replacements or other mutations in gene regions linked to antibiotic resistance," (page 9870, bottom right), and that "greater than 95% of nucleotide changes were directly

associated with antibiotic resistance." (*Id.*, page 9873, left column, second full paragraph.) The authors concluded that "[t]he lack of allelic diversity means that when amino acid polymorphisms, or regulatory region nucleotide variation are observed, there should be strong suspicion that the variation has functional consequences, such as antibiotic resistance." *Id.*, at page 9872, bottom right hand column.

The results in the present specification show that every mutation in the EtaA gene was associated with increased resistance to ETA and to TA, and all but one was associated with increased resistance to TC. The recent report by Morlock et al., provided with this Amendment, shows that every isolate studied that had a mutation in EtaA had increased resistance to ETA. Accordingly, the results reported by Sreevatsan, by the Applicants, and by Morlock, are consistent and show that there is a high degree of predictability that mutations in the EtaA gene cause resistance to ETA, TA, and TC. To expedite prosecution, however, the claims have been amended to recite the particular mutations set forth in the specification.

7. Conclusion

In short, Applicants maintain that the Action has not made out a prima facie case of unpredictability. Every single mutation found thus far in Mtb EtaA, by Applicants or by other researchers, has been correlated with increased resistance to ETA, and no mutation has been reported that is not associated with increased resistance to ETA. Accordingly, Applicants maintain that it is predictable that mutations in the EtaA gene are correlated with resistance to thioamide drugs, and highly correlated with thiocarbonyl drugs in general. Nonetheless, to expedite prosecution, the claims have been amended to recite the mutations set forth in the specification. As amended, the claims are believed to be free of the rejections posed by the Action.

B. Rejection of the Claims As Obvious

Claims 25 remains rejected, and claim 46 is newly rejected, under 35 U.S.C. § 103(a) as obvious over an unpublished sequence deposited by Badcock and Churcher (hereafter,

"Badcock"), in view of Philipp et al., PNAS USA 93:3132-3137 (1996) ("Philipp") and further in view of Ahern, The Scientist, 1995 ("Ahern"). Applicants traverse.

As noted in Applicants' last amendment, Badcock is an unpublished sequence of 38230 nucleotides from the genome of Mtb, nucleotides 14983 to 16452 of which are stated encode a probable monooxygenase. Philipp is a report of an integrated map of the Mtb genome. Ahern is simply an article on the advantages of kits. According to the Action, Badcock teaches the EtaA gene of Mtb and its possible function as a monooxygenase. Action, at page 10. Philipp is stated to establish an ordered set of DNA fragments and supposedly, to teach using primers specific to the Mtb sequence of genomic DNA to facilitate gene mapping.

As noted, the rejection under §103(a) relies on combining Badcock, Philipp and Ahern. Applicants pointed out in their last Amendment that a combination of references requires the Action to show some teaching, suggestion, or motivation in the art to combine the references. *See, e.g., In re Geiger*, 815 F.2d 686 (Fed. Cir. 1987). As the Federal Circuit has noted, "[c]ombining prior art references without evidence of such a suggestion, teaching, or motivation simply takes the inventor's disclosure as a blueprint for piecing together the prior art to defeat patentability--the essence of hindsight. *See, e.g., Interconnect Planning Corp. v. Feil,* 774 F.2d 1132, 1138, 227 USPQ 543, 547 (Fed. Cir. 1985) ("The invention must be viewed not with the blueprint drawn by the inventor, but in the state of the art that existed at the time.")." *In re Dembiczak*, No. 98-1498 (Fed. Cir., April 28, 1999). Applicants maintained in their last Amendment that the previous Action had failed to show that there was any motivation to combine the references as the Action had, absent the teachings of the specification.

The present Action responds by asserting that a motivation to combine the references is in fact present. At page 16, the Action cites a line in Phillip, at page 3137, that "the goal of this study was to elucidate the genomic organization of *M. tuberculosis* and to establish a set of ordered DNA fragments, a valuable genetic resource." This is of course true, but has little obvious relevance to providing motivation to make primer pairs which specifically amplify EtaA, as recited in the claims under examination. Elucidating the genomic organization, and

establishing a set of ordered DNA fragments provides no apparent reason to create primers specific for any of the particular genes in the Mtb genome, let alone the EtaA gene.

The Action quotes Phillip as further teaching that "several recent examples leading to the identification of genes involved in drug resistance or encoding new therapeutic targets testify to the power of this approach." This statement, however, is taken out of context and has nothing to do with amplification of DNA sequences, let alone the amplification of EtaA, in particular. The sentence preceding the one quoted by the Action states: "The clones based on the shuttle vector pYUB18 should facilitate the dissection of the pathogenicity of the tubercule bacillus, as they can be introduced into easily manipulated surrogate hosts, such as *Mycobacterium smegmatis*, where faithful gene expression can be obtained." [Philipp, at page 3137, left column. Emphasis added, citation omitted]. This sentence is then followed by the one quoted by the Action: "Several recent examples [citations omitted] leading to the identification of new genes involved in drug resistance or encoding new therapeutic targets testify to the power of this approach." Id. The "approach" referred to by Philipp, therefore, is the introduction of clones into surrogate hosts and the observation of the phenotype of the resulting organisms. The comments in Philipp therefore do not pertain to the amplification of ordered DNA fragments in general or to primers specific for amplifying the EtaA gene in particular, and would not lead the person of skill to combine the teachings of Philipp with references pertaining to PCR. Philipp therefore does not provide the motivation relied on by the Action for combining the references.

Finally, the Action asserts that "the reference [Philipp] teaches the use of PCR amplification using primers specific to the *Mycobacterium tuberculosis* sequence of genomic DNA in order to facilitate gene mapping, data handling and analysis (Pg. 3133). The examiner maintains that a motivation to combine the references thus exists." Action, at pages 16-17, bridging sentence.

Once again, the portion of Philipp quoted is taken out of context. What Philipp says, at the place cited, is:

Hybridization, Mapping, Data Handling and Analysis

To facilitate gene mapping and the construction of contigs by hybridization mapping [citation omitted], arrays of cosmids were dot blotted onto Hybond N membranes and processed as recommended by Amersham, Plc. The probes were macrorestriction fragments isolated from pulsed-field gels and labeled in the gel slice, whole cosmids, repetitive sequences, or single-copy probes produced either from cloned genes or by PCR amplification of genomic DNA [citation omitted].

The Action asserts that this passage provides motivation to combine the references. But what the passage says is no more than that the gene mapping and data handing were facilitated by immobilizing cosmids on membranes and probing the membranes with probes, which could be single-copy probes produced from cloned genes or by PCR amplification of genomic DNA. There is no indication that any particular portion of genomic DNA or that any particular gene would be any more useful than any other for probing immobilized cosmids on a membrane. Certainly, the motivation to combine the references allegedly present in the passage is not explained by the Action, which merely states a conclusion without presenting any reasoning or analysis to support it.

The Action provides no analysis or reasoning why the practitioner would take the general disclosure of Philipp about probing cosmids with amplified genomic DNA and combine it with Badcock's unpublished disclosure of a probable monooxygenase encoded by 1400 nucleotides of a reported 38,000 nucleotide sequence to render obvious kits with primers specific for that 1400 nucleotides. The Action merely states it as a conclusion, and leaves it to the reader to deduce the reasons that might provide such a motivation.

An unsupported conclusion, however, does not meet the Office's burden to show the logic for a combination. As Applicants pointed out in their last Amendment, the Federal Circuit requires that the examiner: "show reasons that the skilled artisan, confronted with the same problems as the inventor and no knowledge of the claimed invention, **would select the elements from the cited prior art references for combination in the manner cited.**" *In re Rouffet*, 47 USPQ2d 1453 (1998) (Emphasis added.)

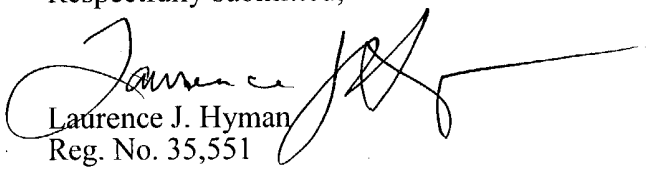
The present Action, like the previous Action, fails to show "reasons that the skilled artisan, confronted with the same problems as the inventor and no knowledge of the claimed invention, would select the elements from the cited prior art references for combination in the manner cited," as required by *Rouffet*. In the absence of such a showing, the conclusion is once again warranted that the Action has simply picked and chosen from the art to recreate the invention. Applicants therefore respectfully submit that the Action has once again failed to present a proper *prima facie* case of obviousness. The rejection should be reconsidered and, upon reconsideration, withdrawn.

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,



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ethA, *inhA*, and *katG* Loci of Ethionamide-Resistant Clinical *Mycobacterium tuberculosis* Isolates

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Ethionamide (ETH) is a structural analog of the antituberculosis drug isoniazid (INH). Both of these drugs target InhA, an enzyme involved in mycolic acid biosynthesis. INH requires catalase-peroxidase (KatG) activation, and mutations in *katG* are a major INH resistance mechanism. Recently an enzyme (EthA) capable of activating ETH has been identified. We sequenced the entire *ethA* structural gene of 41 ETH-resistant *Mycobacterium tuberculosis* isolates. We also sequenced two regions of *inhA* and all or part of *katG*. The MICs of ETH and INH were determined in order to associate the mutations identified with a resistance phenotype. Fifteen isolates were found to possess *ethA* mutations, for all of which the ETH MICs were ≥50 μg/ml. The *ethA* mutations were all different, previously unreported, and distributed throughout the gene. In eight of the isolates, a missense mutation in the *inhA* structural gene occurred. The ETH MICs for seven of the InhA mutants were ≥100 μg/ml, and these isolates were also resistant to ≥8 μg of INH per ml. Only a single point mutation in the *inhA* promoter was identified in 14 isolates. A *katG* mutation occurred in 15 isolates, for which the INH MICs for all but 1 were ≥32 μg/ml. As expected, we found no association between *katG* mutation and the level of ETH resistance. Mutations within the *ethA* and *inhA* structural genes were associated with relatively high levels of ETH resistance. Approximately 76% of isolates resistant to ≥50 μg of ETH per ml had such mutations.

Multidrug-resistant tuberculosis (MDR-TB) is an emerging public health crisis in many regions of the world, particularly in developing nations (29). Tubercle bacilli isolated from patients with MDR-TB are resistant to at least isoniazid (INH) and rifampin (8). These two drugs represent half the standard four-drug tuberculosis treatment regimen along with pyrazinamide (PZA) and either ethambutol or streptomycin (2). These five compounds are referred to as the "first-line" antituberculosis drugs. Infection with MDR-TB complicates tuberculosis treatment by necessitating the selection of substitute drugs, collectively referred to as "second-line" drugs, to replace the ineffective first-line drugs (8). Ethionamide (ETH), one of the most frequently used and efficacious second-line drugs (8), is a structural analog of INH (5). Both compounds are known to inhibit mycolic acid biosynthesis (28). The existence of partially cross-resistant phenotypes has long been known (6, 14, 18). Low-level INH-resistant strains frequently display low-level ETH resistance, while high-level INH-resistant strains typically remain ETH susceptible (6). The structural similarity and existence of cross-resistant phenotypes suggested that these two drugs share a common molecular target (1).

The molecular genetics of INH resistance in *Myobacterium tuberculosis* has been extensively investigated. INH is classified as a prodrug, meaning that it must undergo in vivo transformation to an active form. *katG*-encoded catalase-peroxidase (KatG) performs this function in *M. tuberculosis* (30), and mutations in *katG*, particularly at codon 315, confer INH, but not ETH, resistance (5). The primary target of activated INH is an NADH-dependent enoyl-acyl carrier protein reductase, designated InhA (16). Mutations within the *inhA* structural gene (1, 3, 15, 23) or within the *inhA* promoter (15, 17, 21, 22, 24) have been identified and are associated with both INH and ETH resistance (16, 17). Missense mutations within the *inhA* structural gene cause INH resistance by reducing the NADH binding affinity of InhA and thus protecting the enzyme from INH inactivation (25). The *inhA* promoter mutations upregulate target expression, producing INH and ETH resistance via a drug titration mechanism (1, 16). The structural similarity and shared molecular target of INH and ETH led to the hypothesis that ETH must, like INH, undergo cellular activation (4).

Recently two groups have reported the discovery of an enzyme capable of activating ETH (4, 10). Both groups initially identified a protein that when overexpressed produced ETH resistance. This protein showed homology with members of the TetR family of transcriptional regulators (4, 10). The open reading frame (ORF) encoding this protein is designated Rv3855 in the *M. tuberculosis* genome database. An adjacent, transvergently transcribed ORF (Rv3854c), separated from the other by a 76-bp intergenic region, encodes a protein with homology to known monooxygenases (4, 10). Overexpression of Rv3854c in *Mycobacterium smegmatis* resulted in substantially increased ETH sensitivity relative to wild-type *M. smegmatis* (4, 10). Mycolic acid synthesis was also dramatically inhibited in the Rv3854c expression construct (4). Attempts to overexpress Rv3854c in *M. tuberculosis* were unsuccessful (10).

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TABLE 1. Oligonucleotide primers used in this study

Name	Sequence (5′→3′)[a]	Application		Accession no.
		PCR	Sequencing	
ethA-1	ATC ATC GTC GTC TGA CTA TGG	Yes	Yes	z83864
ethA-2	CGA CAG ACA AAC TCC GAC ACC	No	Yes	z83864
ethA-3	CCT CCT GGA CGC TGA AG	No	Yes	z83864
ethA-4	CCT CGA CCT TCC CGT GA	Yes	Yes	z83864
ethA-5	ACT ACA ACC CCT GGG ACC	Yes	Yes	z83864
ethA-6	CTT GGC GCC CGA GTC C	No	Yes	z83864
ethA-7	CGT CGT GAT CGG CAG TGG C	No	Yes	z83864
ethA-8	GGT GGA ACC GGA TAT GCC TG	Yes	Yes	z83864
ethA-9	CCT CGA GTA CGT CAA GAG CAC	Yes	Yes	z83864
ethA-10	CGT TGA CGG CCT CGA CAT TAC	Yes	Yes	z83864
katG-1	TGG CCG CGG CGG TCG ACA TT	Yes	Yes	x68081
katG-2	CCA GCA GGG CTC TTC GTC AG	Yes	Yes	x68081
inhA-1	CCT CGC TGC CCA GAA AGG GA	Yes	Yes	u41388
inhA-2	ATC CCC CGG TTT CCT CCG GT	Yes	Yes	u41388
inhA-3	AGG TCG CCG GGG TGG TCA GC	Yes	Yes	u41388
inhA-4	AGC GCC TTG GCC ATC GAA GCA	Yes	Yes	u41388

[a] Underlined sequences are in the reverse complement orientation relative to the GenBank sequence.

These results led both groups to conclude that Rv3854c activates ETH and that this enzyme is under the regulatory control of Rv3855. The DeBarber group designated *Rv3855* and *Rv3854c* as *etaR* and *etaA*, respectively, while the Baulard group used the designations *ethR* and *ethA*, respectively.

The identification and characterization of the *ethAR* loci represent a significant advance in understanding the biochemistry of ETH and the mechanistic relationship of this drug to its structural analog, INH. ETH must undergo activation via an EthA-mediated process in a manner analogous to the KatG activation of INH. The putative final metabolites for both drugs are very similar, and they share the same cellular target, namely InhA. Genetic alterations leading to reduced EthA activity would be expected to result in increased ETH resistance, just as *katG* mutations confer INH resistance. The *ethA* genes of 11 ETH-resistant isolates were sequenced, and coding sequence mutations were found in all of them (10).

While the ability of EthA to activate ETH has been convincingly demonstrated, very limited data exist on the occurrence of *ethA* mutations in ETH-resistant *M. tuberculosis* clinical isolates. This investigation was undertaken to provide additional data regarding the relative prevalence of *ethA* and *inhA* mutations in such isolates. We sequenced either all or part of the *ethA*, *inhA*, and *katG* genes of 41 ETH-resistant clinical isolates of *M. tuberculosis*. To evaluate the relative phenotypic impact of mutations within these genes, we determined the MICs of ETH and INH for all isolates.

MATERIALS AND METHODS

Mycobacterial strains and genomic DNA isolation. The 41 isolates of *M. tuberculosis* examined in this study were obtained from patients in the United States, Russia, and Brazil. The U.S. isolates (n = 29) had been submitted to the Centers for Disease Control and Prevention for routine drug susceptibility testing. The Russian isolates (n = 6) had been collected as part of a drug resistance surveillance study and originated from the oblasts of Ivanovo and Vladimir. The Brazilian specimens (n = 6) were obtained from a collection of isolates gathered for a study of the molecular genetics of INH resistance in Brazil. All isolates were resistant to 10 μg of ETH per ml when tested by the method of proportions (7) with Middlebrook 7H10 agar. Isolates were stored frozen at −70°C until selected for this study.

Genomic DNA was prepared by a minibead cell disruption protocol. One milliliter of a 2-week-old 7H9 broth culture was added to a 2.0-ml screw-cap microcentrifuge tube containing Lysing Matrix B (Qbiogene, Inc., Carlsbad, Calif.). The tubes were then incubated for 20 min at 95°C to kill the cells. Next, 200 μl of chloroform and 300 μl of Tris-EDTA (TE) buffer were added to each tube. This mixture was vigorously agitated for 1 min with a Mickle cell disrupter (Brinkman Instruments, Inc., Westbury, N.Y.) and then centrifuged at 10,000 rpm for 5 min. The aqueous phase, which contained genomic DNA, was collected and stored at 4°C.

DNA amplification and sequencing. The entire *ethA* ORF was PCR amplified. Because of the large size of this ORF (1,470 bp), three reactions were performed for each sample, producing three overlapping PCR amplicons: ETH1, ETH2, and ETH3. The primers used and the sizes of the amplicons generated are as follows: primers ethA-1 and ethA-5 produced a 667-bp product designated ETH1, primers ethA-4 and ethA-9 produced a 692-bp product (ETH2), and primers ethA-8 and ethA-10 produced a 342-bp product (ETH3). All reaction mixtures contained 12.5 μl of HotStartTaq master mix (Qiagen Inc., Santa Clarita, Calif.), 1.0 μl of template DNA, and each primer at a final concentration of 0.3 μM. Each reaction was adjusted to a final volume of 25 μl with Type I water. The amplification profile for ETH1 and ETH2 consisted of an initial 15-min denaturation and enzyme activation at 95°C followed by 35 cycles of 95°C denaturation for 30 s and 65°C annealing and elongation for 1.25 min and a final 5-min elongation. The profile for ETH3 was identical, except that the annealing and elongation temperature was 68°C. All amplifications were performed in a Gene-Amp PCR system 2400 thermal cycler (Perkin-Elmer, Inc., Foster City, Calif.).

A 322-bp fragment of *katG* encompassing codon 315 was PCR amplified with primers katG-1 and katG-2. A 248-bp fragment containing the *inhA* promoter was amplified with primers inhA-1 and inhA-2. Nucleotide residues 13 to 379 of the 810-nucleotide (nt) *inhA* ORF were amplified with primers inhA-3 and inhA-4. The PCR mixtures and thermal cycler used to amplify these three loci were the same as those described for *ethA* amplification. All three loci were amplified with the same thermal cycling profile of 15 min of denaturation and enzyme activation at 95°C followed by 35 cycles of 95°C denaturation for 30 s, 60°C annealing for 30 s, 72°C elongation for 30 s, and a final 5-min elongation. The entire *katG* structural gene was sequenced in a select subset (n = 12) of the isolates.

Automated DNA sequencing was performed by dichlororhodamine BigDye terminator chemistry (Perkin-Elmer, Inc.). The protocol supplied by the manufacturer was modified by halving the volume of master mix used and adjusting the ionic strength of the reaction mixture with 5× sequencing buffer (Perkin-Elmer, Inc.). The fluorescent elongation products were electrophoresed on a model 373XL DNA sequencer (Perkin-Elmer, Inc.). Amplicons ETH1 and ETH2 of *ethA* were each sequenced with four internal primers and the *katG*, *inhA* promoter, *inhA* ORF, and ETH3 amplicons were sequenced with the primers used for amplification. A complete list of all PCR and sequencing primers, with their nucleotide sequences, is found in Table 1. All *ethA* primers were designed with Oligo version 6.0 primer analysis software (Molecular Biology Insights, Inc.,

Cascade, Colo.). Sequence analyses were performed with Sequence Navigator version 1.0.1 software (Perkin-Elmer, Inc.), and all sequencing runs included the pan-susceptible strain *M. tuberculosis* $H_{37}Rv$ (ATCC 27294) as a wild-type control. Each sequence was compared with that of both the control strain and the appropriate published sequence.

Strain differentiation. All *M. tuberculosis* isolates were genotyped by the spoligotyping method (19). Each spoligotype pattern was assigned an octal code as described by Dale et al. (9).

ETH and INH susceptibility testing. ETH and INH susceptibility testing was performed with the microplate alamar blue assay (MABA) (12). A 32-mg/ml stock solution of ETH (Sigma Chemical Co., St. Louis, Mo.) was prepared in dimethyl sulfoxide (DMSO; J. T. Baker, Inc., Phillipsburg, N.J.), and a 10-mg/ml stock solution of INH (Sigma Chemical Co.) was prepared in sterile water. The stock solutions were aliquoted and stored at −70°C. The ETH stock was diluted with Middlebrook 7H9 broth to a concentration of 400 μg/ml, and the solution was subsequently twofold serially diluted, resulting in solutions ranging in concentration from 400 to 50 μg/ml. Four additional twofold serial dilutions ranging in concentration from 40 to 5.0 μg/ml were formulated by the same approach.

The perimeter wells of 96-well, clear microtiter plates (Costar 3596; Corning, Inc., Corning, N.Y.) were filled with 200 μl of sterile water to prevent the plates from drying out during incubation. The wells in rows B through F of each column received 100 μl of test medium, with the drug concentration highest in column 2 and diminishing in order through column 9. Columns 10 and 11 received 100 and 200 μl of drug-free medium, respectively. To control for any possible inhibitory effect of the DMSO on cell growth, the wells in row G received 100 μl of DMSO-containing media. The concentration of DMSO in each column was equivalent to that of the ETH-containing wells in that column. The INH assay plate was prepared in a similar manner, with concentrations ranging from 64 to 0.5 μg/ml.

The *M. tuberculosis* strains were cultured for 2 weeks, after which the turbidity was visually adjusted with 7H9 broth to that equivalent to a McFarland no. 1 standard. The inocula were prepared by diluting the standardized cultures 1:25 with 7H9 broth. Each test well received 100 μl of inoculum. The wells in column 11 were not inoculated and served as a sterility control. The plates were sealed with transparent tape. The pan-susceptible *M. tuberculosis* strains $H_{37}Rv$ and "circle 8" (a clinical isolate routinely used as a control in our laboratory) were used as controls.

The plates were incubated at 35°C under ambient conditions. The detection reagent was prepared by diluting a 10× alamar blue (Trek Diagnostic Systems, Inc., Westlake, Ohio) solution 1:1 with freshly prepared 10% Tween 80 (Sigma Chemical Co.). After 9 days of incubation, 25 μl of alamar blue solution was added to the drug-free control wells (columns 10 and 11). The plates were incubated for another 24 h, after which the control wells were examined. A color change from blue to pink in the inoculated wells and no color change in the uninoculated wells validated the controls. Once the controls were validated, 25 μl of alamar blue solution was dispensed into the remaining test wells. The plates were examined, and results were recorded after another 24 h of incubation. The MIC was defined as the lowest drug concentration that prevented a color change. All strains were tested in duplicate on separate occasions.

Disclaimer. Use of trade names is for identification only and does not constitute endorsement by the U.S. Department of Health and Human Services, the Public Health Service, or the Centers for Disease Control and Prevention.

RESULTS

DNA sequencing of *ethA*, *inhA*, and *katG* loci. A complete list of specific mutations identified as well as the MICs of ETH and INH for each isolate is provided in Table 2. The entire *ethA* ORFs of the 41 *M. tuberculosis* isolates were sequenced. An *ethA* mutation was identified in 15 (37%) of those isolates; all 15 mutations were unique and had not been previously reported. The mutations were distributed from nt 110 through 1387 of the 1,470-nt ORF. The types of mutations included eight missense mutations, 1 nonsense mutation, four 1-nt deletions, one 2-nt deletion, and one 1-nt insertion. Nine of the *ethA* mutants, including all six of the frameshift mutants, had no *inhA* mutations, while the other six also had *inhA* regulatory mutations. The ETH MICs for the *ethA* mutants ranged from 50 to ≥200 μg/ml.

Two separate regions of the *inhA* gene, one encompassing codons 13 to 119 of the ORF (270 total codons) and another containing the putative promoter, were examined in all isolates. Mutations in the *inhA* ORF were identified in 9 (22%) of the 41 isolates. Among the nine *inhA* ORF mutants, four different point mutations were identified: three were missense (Ile 21→Thr or Val and Ser 94→Ala) and one was silent (Leu 44→Leu). Twenty-seven (66%) of the 41 isolates displayed a mutation in the *inhA* regulatory region. All but one of these involved the substitution of cytosine for thymine at the position 15 nt upstream (−15) of the *mabA* initiation codon. The one exception was a thymine-to-cytosine transition at position −17. Six of the eight *inhA* ORF missense mutants also displayed *inhA* regulatory mutations. Only one isolate (isolate 15) possessed mutations in all of the regions examined; however, the *inhA* ORF mutation was silent. Three isolates were wild type at both the *ethA* and *inhA* loci, two of which were susceptible to the lowest concentration of ETH tested, while the MIC for the third isolate was 50 μg/ml.

The complete *katG* ORF of 12 isolates and a fragment encompassing codons 249 through 342 (741 total codons) of the remaining isolates were sequenced. Mutations in *katG* were found in 15 (37%) of the 41 isolates. Eleven of those mutants had a guanine-to-cytosine transversion at nt 944, resulting in the substitution of threonine for serine at amino acid residue 315. The four other *katG* mutations identified were Asn 138→Thr, Glu 195→Lys, Gly 279→Asp, and Trp 341→Ser. The INH MICs for all but one of the *katG* mutants were ≥32 μg/ml: the INH MIC for the Glu 195→Lys mutant (isolate 35) was 1 μg/ml. Tables 3 and 4 list the number of isolates with mutations in each locus, or combination of loci, stratified according to MIC.

Strain differentiation. Twenty-eight different spoligotype patterns were identified among the 41 study isolates. Twenty-three of these patterns were represented by a single isolate, and the remaining 18 isolates were distributed among five clusters. The largest of these five clusters included eight isolates, one cluster contained four isolates, and three clusters consisted of two isolates each. Among the clustered isolates, none had mutation profiles identical to those of the other members of its cluster. All five of the single-base frameshift mutants were members of the eight-isolate cluster. The spoligotype of each of the isolates is shown in Table 2.

ETH and INH MICs. We determined the MICs of ETH and INH for all 41 isolates in order to compare the types of mutations identified with drug resistance levels. The INH MICs showed perfect concordance between replicates in 39 (95%) of the isolates. The MICs of the two discrepant isolates differed by only 1 dilution. The end points for INH were sharp and unambiguous, as indicated by the absence of a color change from blue to pink in the MIC well. The end points for ETH were somewhat less obvious, since color changes were gradual as the end point was approached. This "trailing" effect typically occurred over 1 to 2 drug dilutions, but did not occur for isolates that were either fully susceptible or resistant to ≥200 μg of ETH per ml. To confirm our 24-h readings, we allowed the plates to incubate for an additional 24 h, after which time, the color change from blue to pink became more pronounced. The ETH MICs for 27 (66%) of the isolates were identical between tests: 10 (24%) differed by 1 dilution, and 4 (10%)

TABLE 2. Characteristics of 41 clinical *M. tuberculosis* isolates

Isolate no. (origin)[a]	Spoligotype	MIC (μg/ml)		Genetic alteration						
				ethA		*katG*[b]		*inhA* ORF		*inhA* regulator (nucleotide no.)
		ETH	INH	Nucleotide no.	Amino acid	Nucleotide no.	Amino acid	Nucleotide no.	Amino acid	
1 (US)	677777477413771	>200	>32	None	NA[c]	**None**	**NA**	T → C62	I21 → T	C (−15) T
2 (US)	000000000003771	>200	8	None	NA	**None**	**NA**	T → G280	S94 → A	None
3 (US)	477777777413771	>200	4	None	NA	None	NA	None	NA	C(−15) T
4 (US)	000000000000771	>200	8	C → T736	Q246 AMB	None	NA	None	NA	C(−15) T
5 (US)	777737776413731	>200	2	None	NA	None	NA	None	NA	C(−15) T
6 (US)	667777477413771	>200	8	None	NA	**None**	**NA**	A → G61	I21 → V	C (−15) T
7 (US)	677777477413771	>200	8	None	NA	**None**	**NA**	A → G61	I21 → V	C (−15) T
8 (R)	000000000003771	>200	>32	T deleted at 703	Frameshift	G → C944	S315 → T	None	NA	None
9 (R)	000000000003771	>200	32	A deleted at 110	Frameshift	G → C944	S315 → T	None	NA	None
10 (R)	000000000003771	>200	>32	G deleted at 768	Frameshift	G → C944	S315 → T	None	NA	None
11 (US)	647777477413771	>200	4	None	NA	None	NA	None	NA	C (−15) T
12 (BZ)	777777777760601	>200	32	A → G1174	T392 → A	G → C944	S315 → T	None	NA	None
13 (BZ)	777741017760771	>200	>32	G → A1154	G385 → D	G → C944	S315 → T	None	NA	C (−15) T
14 (BZ)	677737607760771	>200	>32	None	NA	**None**	**NA**	T → C62	I21 → T	C (−15) T
15 (BZ)	777777777760731	>200	>32	A → C167	D55 → A	G → A836	G279 → D	C → T130	L44 → L	C (−15) T
16 (US)	777777777760740	>200	0.5	GC deleted at 1322–1323	Frameshift	None	NA	None	NA	None
17 (US)	777777776413771	>200	>32	None	NA	**None**	**NA**	None	NA	C (−15) T
18 (R)	777777607760771	200	>32	T → G1013	I338 → S	G → C944	S315 → T	None	NA	C (−15) T
19 (US)	000000000003071	200	32	G → A127	G43 → S	G → C944	S315 → T	None	NA	None
20 (US)	777777774020771	100	8	None	NA	**None**	**NA**	T → G280	S94 → A	C (−15) T
21 (US)	777777777720771	100	8	None	NA	**None**	**NA**	T → C62	I21 → T	G (−17) T
22 (US)	000000000003771	100	32	A inserted at 338	Frameshift	G → C944	S315 → T	None	NA	None
23 (US)	677777477413771	100	2	None	NA	None	NA	None	NA	C (−15) T
24 (US)	700777747413771	100	2	None	NA	None	NA	None	NA	C (−15) T
25 (US)	000000000003771	50	32	C deleted at 1290	Frameshift	G → C944	S315 → T	None	NA	None
26 (US)	777777777760771	50	2	G → A668	E223 → K	None	NA	None	NA	C (−15) T
27 (US)	474377777413771	50	2	G → A1238	G413 → D	**None**	**NA**	None	NA	None
28 (US)	477777777413771	50	2	None	NA	**None**	**NA**	None	NA	None
29 (BZ)	777777607760771	50	>32	C → A1387	R463 → S	G → C1022	W341 → S	None	NA	C (−15) T
30 (R)	000000000003771	25	2	None	NA	None	NA	None	NA	C (−15) T
31 (US)	777777777760771	20	2	None	NA	None	NA	None	NA	C (−15) T
32 (US)	677777477413751	20	2	None	NA	None	NA	None	NA	C (−15) T
33 (US)	776377760000731	20	4	None	NA	None	NA	None	NA	C (−15) T
34 (US)	700036777760711	20	2	None	NA	None	NA	None	NA	C (−15) T
35 (US)	677777477413771	10	1	None	NA	**G → A583**	**E195 → K**	T → G280	S94 → A	None
36 (US)	777777777740711	10	2	None	NA	None	NA	None	NA	C (−15) T
37 (US)	776000003660771	10	2	None	NA	None	NA	None	NA	C (−15) T
38 (BZ)	677777607760771	10	>32	None	NA	G → C944	S315 → T	None	NA	C (−15) T
39 (US)	000000007760771	<2.5	2	None	NA	None	NA	None	NA	C (−15) T
40 (US)	777777774020771	<2.5	>32	None	NA	**A → C413**	**N138 → T**	None	NA	None
41 (R)	000000000003771	<2.5	>32	None	NA	G → C944	S315 → T	None	NA	None
$H_{37}Rv$	777777477760771	<2.5	0.5	None	NA	None	NA	None	NA	None
circle 8	777777774020771	<2.5	<0.25	None	NA	None	NA	None	NA	None

[a] Country where the isolate was collected. US, United States; R, Russia; BZ, Brazil.
[b] Results in boldface indicate the entire *katG* ORF was sequenced.
[c] NA, not applicable.

differed by 2 dilutions. In all cases, we defined the MIC as the lowest concentration that prevented any color change compared with the negative control.

The ETH MICs for 15 isolates with *ethA* mutations, either alone (n = 9) or in combination with *inhA* promoter mutations, were ≥50 μg/ml. No isolate for which the ETH MIC was ≤25 μg/ml possessed *ethA* mutations. Six isolates displayed both *inhA* ORF and promoter mutations, and the ETH MIC for all of them was ≥100 μg/ml. Two strains with Ser 94→Ala substitutions in InhA had a wild-type promoter; one of these (isolate 2) was wild type at all other loci examined, while the other (isolate 35) also had a Glu 195→Lys substitution in *katG*. Isolate 35 was much less resistant to both ETH and INH than was isolate 2.

In 14 of the isolates, only *inhA* promoter mutations were identified. The ETH MICs for these isolates varied greatly, with six being resistant to ≥100 μg/ml, seven being in the range of 10 to 25 μg/ml, and one being susceptible to the lowest concentration tested. The INH resistance level of these isolates was much more consistent, with INH MICs for 13 being either 2 or 4 μg/ml. The sole exception (isolate 17) was resistant to >32 μg of INH per ml. This strain was also resistant to 200 μg of ETH per ml.

The INH MICs for 17 (41%) of the study isolates were ≥32 μg/ml. Fourteen of these possessed *katG* mutations, either alone (n = 9) or in combination with *inhA* promoter mutations (n = 5). Two strains (isolates 1 and 14) resistant to 32 μg of INH per ml had both an Ile 21→Thr substitution in InhA and

TABLE 3. ETH MICs for and mutations in the *ethA* and *inhA* loci of 41 clinical *M. tuberculosis* isolates

ETH MIC (μg/ml)	No. of isolates	No. of isolates with mutations in[a]:					No. of isolates with no mutation
		ethA only	*inhA*			*ethA* and *inhA* promoter	
			ORF only	Promoter only	ORF and promoter		
>200	17	5	1	4	4	3	0
200	2	1	0	0	0	1	0
100	5	1	0	2	2	0	0
50	5	2	0	0	0	2	1
25	1	0	0	1	0	0	0
20	4	0	0	4	0	0	0
10	4	0	1	3[c]	0	0	0
5	0	0	0	0	0	0	0
2.5	0	0	0	0	0	0	0
<2.5	3	0	0	1	0	0	2[e]
Total	41	9[b]	2	15	6	6[d]	3

[a] Exclusive of mutations in *katG* and silent mutations.
[b] Includes seven isolates with *katG* mutations.
[c] Includes one isolate with a *katG* mutation.
[d] Includes four isolates with *katG* mutations.
[e] Both isolates have *katG* mutations.

an *inhA* promoter mutation. The INH MICs for four strains with both *inhA* ORF and promoter mutations, one strain with only an ORF mutation (isolate 2), and one strain with only a promoter mutation (isolate 4) were 8 μg/ml. At least one example of each of the three InhA substitutions identified in this study is represented in this group. In the case of the Ile 21→Thr substitution, this change occurred in conjunction with a point mutation within the *inhA* promoter, resulting in the replacement of guanine with thymine at position −17. This contrasts with two strains resistant to 32 μg of INH per ml that had the same *inhA* ORF mutation but in combination with the −15 promoter mutation. The INH MIC for one isolate (isolate 35) with a Ser 94→Ala substitution in InhA and a Glu 195→Lys substitution in KatG was 1 μg/ml. No INH resistance-associated mutations were identified in four INH-resistant isolates.

TABLE 4. INH MICs for and mutations in the *katG* and *inhA* loci of 41 clinical *M. tuberculosis* isolates

INH MIC (μg/ml)	No. of isolates	No. of isolates with mutations in[a]:					No. of isolates with no mutation
		katG only	*inhA*			*katG* and *inhA* promoter	
			ORF only	Promoter only	ORF and promoter		
>32	12	4	0	1	2	5[c]	0
32	5	5	0	0	0	0	0
16	0	0	0	0	0	0	0
8	6	0	1	1[d]	4	0	0
4	3	0	0	3	0	0	0
2	13	0	0	11[d]	0	0	2[f]
1	1	1[b]	0	0	0	0	0
0.5	1	0	0	0	0	0	1[f]
0.25	0	0	0	0	0	0	0
<0.25	0	0	0	0	0	0	0
Total	41	10[e]	1	16	6	5	3

[a] Exclusive of mutations in *ethA* and silent mutations.
[b] Isolate also has an *inhA* ORF mutation.
[c] Includes seven isolates with *ethA* mutations.
[d] Includes one isolate with an *ethA* mutation.
[e] Includes four isolates with *ethA* mutations.
[f] Includes one isolate with an *ethA* mutation.

DISCUSSION

Our finding of 15 different mutations among 15 *ethA* mutants combined with the 9 previously described mutations suggests that a high degree of genetic diversity occurs within the *ethA* genes of ETH-resistant *M. tuberculosis* isolates. The 15 *ethA* mutants we describe represent 10 spoligotype patterns, including clusters of two and five isolates. All members of the five-isolate cluster had single-nucleotide frameshift mutations. These results indicate that *ethA* mutations appear to be widely disbursed across the structural gene, with no single nucleotide or codon predominating. This distribution contrasts with the situation seen in *katG*, where the majority of the mutations occur at codon 315, with Ser 315→Thr being the most prevalent. The predominance of the codon 315 mutations has been explained by the need for the cell to maintain a minimum basal level of catalase-peroxidase activity to protect against organic peroxides. Alterations that reduce KatG activity below this critical level would be lethal, and changes that lead to little or no reduction in enzyme activity would result in little or no decrease in INH susceptibility.

The fact that no such well-adapted *ethA* mutation has emerged in the ETH-resistant bacilli investigated suggests the existence of one or more enzymes with functional redundancy to EthA. In fact, the genome of *M. tuberculosis* possibly encodes more than 30 monooxygenases (4). The proliferation of such enzymes in *M. tuberculosis* may have evolved as a protective mechanism against various xenobiotic substances (10). The exact role of EthA is not known, but the gene is highly conserved throughout the genus, suggesting it serves an important function (4). Diminution, or loss, of EthA activity would thus be expected to have a deleterious effect on the cell. Given the proliferation of EthA homologs, it seems likely that one or more of these enzymes may be capable of compensating for a loss of EthA activity.

Clearly further study is needed to substantiate the association between *ethA* mutations and ETH resistance and to establish the extent of genetic diversity in this gene. Should the initial finding that a wide array of mutations occur in ETH-resistant strains be verified by future investigation, such a phenomenon would resemble that seen in the *pncA* gene of *M. tuberculosis* strains resistant to PZA (20). This gene encodes pyrazinamidase, the enzyme responsible for the conversion of the PZA into its metabolically active derivative pyrazinoic acid (26). ETH, INH, and PZA are all nicotinamide analogs, and all three drugs rely on fortuitous enzymatic conversion to their respective active metabolites.

The predominance of a single, well-adapted mutation in the *katG* gene of high-level INH-resistant strains reflects this enzyme's critical function of detoxifying reactive oxygen species. Under those rare circumstances in which KatG expression is completely lost, this loss occurs in conjunction with a mutation in the *ahpC* promoter that up-regulates expression of AhpC, an enzyme also involved in antioxidant defense (27). In contrast, PZA-resistant strains display a wide diversity, both in number and spatial distribution, of *pncA* mutations, and no particular mutation predominates. The *ethA* genes of high-

level ETH-resistant strains appear to possess a similar degree of genetic diversity, and no evidence of selective pressure favoring a particular mutation has emerged.

While all of the *ethA* mutants identified were resistant to ≥50 μg of ETH per ml, together they accounted for only 15 (52%) of the 29 isolates displaying that phenotype. An *inhA* missense mutation was found in half of the remaining 14 isolates. Two isolates (isolates 1 and 7) with a shared spoligotype pattern had mutations in adjacent nucleotides of *inhA* codon 21 that resulted in different amino acid substitutions. These two isolates have very different levels of INH resistance. The higher INH MIC for isolate 1 may result from the replacement of an aliphatic isoleucine residue with a weakly polar hydroxyl-containing threonine residue. Such a replacement can produce a greater disruption of InhA structure than is produced when an aliphatic valine residue is substituted, as in isolate 7. X-ray crystallography of InhA has shown that Ile 21 is located in the NADH binding site (11). The fact that no similar disparity was seen in ETH MICs may result from subtle differences in drug-target interactions between ETH, INH, and the InhA-NADH complex. Alternatively, differences in ETH resistance between the two mutants may have gone undetected because they occur at concentrations >200 μg/ml. An Ile 21→Thr substitution occurred in an unrelated strain from Brazil (isolate 14) that was also resistant to >200 μg of ETH per ml and >32 μg of INH per ml, suggesting that the phenotype associated with that particular mutation is consistent across strains. The phenotype associated with the Ile 21→Val substitution also recurred in a second strain (isolate 6); however, this strain differed from the matched pair by one spacer.

InhA structural gene mutations were far more prevalent in this study than in previous investigations. This inconsistency presumably reflects the different criteria used for selecting the study specimens. We selected our isolates on the basis of ETH resistance, whereas in previous investigations, isolates were selected on the basis of INH resistance (21, 22, 24).

We identified a Ser 94→Ala mutation in the *inhA* structural gene of three strains (isolates 2, 20, and 35). This mutation was first described in the seminal paper identifying InhA as the target of ETH and INH (1). Curiously, we are not aware of any prior report describing the originally identified Ser 94→Ala alteration in clinical isolates. In one strain (isolate 2), this was the only mutation identified, while in the others, it occurred in combination with either a *katG* (isolate 35) or an *inhA* promoter mutation (isolate 20). The resistance phenotypes of these strains differed dramatically. The inconsistency of these results is difficult to reconcile but suggests the involvement of other, strain-specific factors.

An *inhA* promoter mutation was identified in 15 isolates with wild-type *ethA* and *inhA* structural genes. The ETH MICs for eight of these isolates were in the range of 10 to 25 μg/ml, a moderate increase in ETH resistance that is consistent with a drug titration mechanism. Four of the 15 promoter mutants were resistant to >200 μg of ETH per ml, and the MIC for 2 mutants each was100 μg/ml. It is highly improbable that the promoter mutation alone can account for the high-level ETH resistance seen in those isolates. This assertion is supported by the fact that the INH MICs for five of these strains were ≤4 μg/ml. Were the promoter mutation alone responsible for the high-level ETH resistance seen in these strains, we would expect a concomitant and proportional increase in INH resistance.

A more plausible explanation for the high-level ETH resistance of those strains is that other, ETH-specific mechanisms of resistance are involved. Expression of EthA is under the negative regulatory control of the protein repressor EthR. An increase in EthR expression would then down-regulate *ethA*, ultimately leading to less drug activation and increased resistance to ETH. Hyperexpression of EthR has been experimentally proven to cause ETH resistance (4, 10) and could therefore explain the highly ETH-resistant phenotype of the six strains with only an *inhA* promoter mutation. How EthR production is controlled and to what stimuli it responds are unknown. The potentially important involvement of *ethR* in clinical ETH resistance shows the need for additional studies to determine which factors mediate EthR production.

The MABA method proved itself a very useful research tool for correlating specific mutations in ETH and INH drug resistance markers with relative resistance phenotypes. The INH MICs obtained were highly reproducible between tests. Establishing a precise end point for ETH was somewhat technically challenging because of "trailing" effect, but there was good reproducibility between replicates. The different end point characteristics of ETH and INH presumably reflect the in vitro bactericidal potency of the two drugs: INH is considered to be bactericidal at or near its MIC, while ETH is bactericidal at concentrations 2 to 4 times its MIC (13). The MICs we report here are specific to the MABA method, and we caution the reader against extrapolating these results to other drug susceptibility testing methods.

In summary, our finding of *ethA* mutations in 52% of clinical isolates for which ETH MICs were ≥50 μg/ml provides substantial new evidence confirming the role of this gene in ETH resistance. As expected, mutations in *ethA* had no detectable association with INH resistance. The level of INH resistance in the study isolates was explainable by and consistent with mutations in *katG* and *inhA*. Twenty-four percent of the high-level ETH-resistant strains had mutations in the *inhA* structural gene. With the exception of Ser 94→Ala, these mutations always occurred in combination with *inhA* promoter mutations. Only an *inhA* promoter mutation was identified in approximately a third of the isolates. The majority of those isolates displayed intermediate levels of ETH and INH resistance. Six of the promoter mutants were resistant to ≥100 μg of ETH per ml, a high level of resistance that we believe is not exclusively attributable to the promoter mutations but rather results from another mechanism. Because the regulatory protein EthR mediates *ethA* expression, it seems reasonable that activator and target mutations alone cannot account for all observed high-level ETH resistance. While mechanisms of ETH resistance exclusive of the *ethAR* loci cannot be discounted, it seems probable that mutation in *ethA* is not the only ETH resistance-associated mechanism involving these loci. While the identification of the *ethAR* loci has contributed greatly to the understanding of ETH resistance, additional investigation is clearly needed.

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